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Reversibility of structural rearrangements in the negative vesicular membrane upon electrostatic adsorption/desorption of the polycation

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Abstract

Interaction of small unilamellar vesicles (SUVs), composed of negative diphosphatidylglycerol (cardiolipin, CL^{2-}) and neutral dipalmitoylphosphatidylcholine (DPPC), with poly(*N*-ethyl-4-vinylpyridinium bromide) (PEVP) was studied in water solution above and below the vesicular membrane melting point by means of differential scanning calorimetry, photon correlation spectroscopy, microelectrophoresis, conductometry, and fluorescence techniques. It has been found that CL^{2-} species are homogeneously distributed within DPPC-CL^{2-} SUV membrane leaflets and between them. Interaction of PEVP with DPPC-CL^{2-} SUVs led to drastic structural rearrangements in the membrane if it was in the fluid state (liquid SUVs). Negative CL^{2-} molecules migrated from the inner to the outer membrane leaflet and segregated in the vicinity of adsorbed PEVP chains. In addition, PEVP adsorption terminated completely the exchange of lipid molecules between the SUVs. At the same time, the integrity of liquid SUVs contacting PEVP remained unchanged. Since the interaction of PEVP with liquid SUVs was predominantly electrostatic in nature, the polycation could be completely removed from the vesicular membrane by addition of an excess of polyacrylic acid (PAA) polyanions forming a more stable electrostatic complex with PEVP. Removal of PEVP resulted in complete resumption of the original distribution of lipids in lateral and transmembrane directions as well as intervesicular lipid exchange. In contrast, PEVP interacting with DPPC-CL^{2-} SUVs formed defects in the vesicular membrane if it was in the gel state (solid SUVs). Such interaction was contributed not only by electrostatic but most likely by hydrophobic interactions involving the defected membrane sites. PEVP kept contacting solid SUVs in the presence of an abundant amount of PAA. The established phenomena may be important for understanding the biological effects of polycations. © 2002 Published by Elsevier Science B.V.

Keywords: Lipid vesicle; Lateral segregation; Flip-flop; Lipid exchange; Polycation; Adsorption; Structural rearrangement; Reversibility

Abbreviations: SUV, small unilamellar vesicle; CL^{2-}, diphosphatidylglycerol (cardiolipin); DPPC, dipalmitoylphosphatidylcholine; FITC-DPPE, *N*-fluorescein isothiocyanyldipalmitoylphosphatidylethanolamine; PEVP, poly(*N*-ethyl-4-vinylpyridinium bromide); PAA, polyacrylic acid; *D*, mean hydrodynamic diameter; EPM, electrophoretic mobility; *v*, molar content of negative CL^{2-} head groups in vesicles; φ, content of a fluorescent lipid in vesicles

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1. Introduction

Synthetic polyelectrolytes are widely used now in medicine and biology. In particular, they have been applied as effective immunostimulants [1–3], DNA-complexing counterparts in constructs for gene delivery [4–8], anticoagulants [9,10], and antiviral [11,12] and antimicrobial agents [13–15]. Such permanently expending biomedical applications stimulate studies of the behavior of polyelectrolytes in biological environment and especially their interaction with cells. Alongside with native cells, cell-mimetic objects, such as spherical bilayer vesicles composed of lipid molecules, can be used for such studies. It is known that adsorption of polyelectrolytes on the cell or vesicular surfaces may cause an increase of the gel-to-liquid–crystalline phase transition temperature [16–18], incorporation of polyelectrolytes into the membrane [19–21], clustering of membrane proteins [22–24] or lipids [16,17,25–27], enhancement of transmembrane migration of lipid molecules (flip-flop) [28,29], and an increase in the permeability of membranes to inorganic ions [30–32]. All these effects, if they occur in biological membranes, may affect the state of the cell as well as its functioning.

Polyelectrolytes or polyelectrolyte-containing constructs introduced into a biological liquid (blood, lymph, etc.) first bind to any proteins or occasional cells. Then, if such random binding is reversible, they would migrate from cell to cell searching by trial and error the targets corresponding to a thermodynamically optimal interaction. In such a case, an important question arises: whether and to what extent do cell membranes restore their structural organization after a polyelectrolyte has been removed? The above aspect remains practically unexplored up to now, in spite of its obvious importance.

In the present work potential consequences of polyelectrolyte–cell interaction were studied using small unilamellar vesicles (SUVs), composed of negative and neutral lipids, namely diphosphatidylglycerol (cardiolipin, CL^{2-}) and dipalmitoylphosphatidylcholine (DPPC), as cell-mimetic species, interacting with a cationic polyelectrolyte, *N*-ethylated poly(4-vinylpyridine) (PEVP).

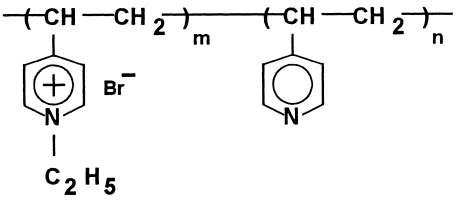
In particular, the following items were investigated: (1) the effect of phase state of the vesicular membrane on the composition and structure of polycation–vesicle complexes; (2) the ability of competing polyanions to remove the polycation from the vesicular membrane; and (3) the reversibility of structural rearrangements in the membrane caused by adsorption/desorption of the polycation.

For this purpose, mixed DPPC-CL^{2-} SUVs were prepared. Their lipid composition was adjusted so that the phase transition of the membrane from gel to fluid state was in the temperature range 20–55°C, which is convenient for experimental studies below and above the transition. Thus we could operate either with solid or liquid vesicles of the same overall lipid composition.

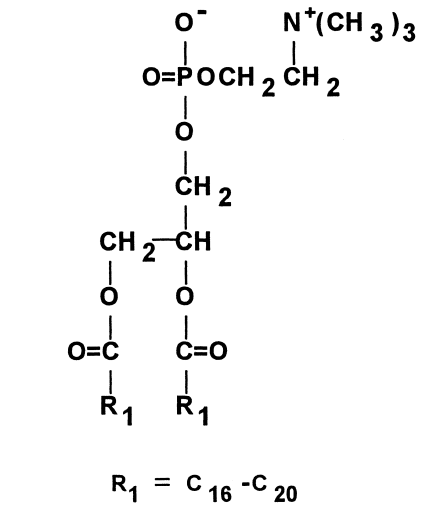
2. Materials and methods

2.1. Materials

PEVP (**I**) was synthesized by quaternization of poly(4-vinylpyridine), degree of polymerization (DP) equal to 1100, as described in [33]. The extent of quaternization was determined by IR spectroscopy measuring the ratio of absorption intensities at 1640 and 1600 cm^{-1} [34]. The resulting product was a copolymer containing 93–95 mol% of vinyl pyridinium and 5–7% of residual 4-vinylpyridine repeating units. Polyacrylic acid (PAA; Aldrich, USA), DP = 70, was used as received. DPPC (**II**), CL^{2-} (**III**), and *N*-fluorescein isothiocyanyldipalmitoylphosphatidylethanolamine (FITC-DPPE) were obtained from Sigma and used as received.

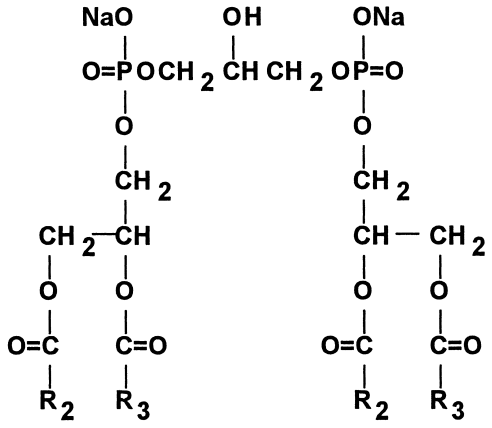


(I)



$R_1 = C_{16}\text{-}C_{20}$

(II)



$R_2 = R_3 = C_{16}\text{-}C_{20}$

(III)

DPPC-CL^{2-} SUVs of 40–60 nm diameter and the molar content of negative CL^{2-} head groups $\nu = 2[CL^{2-}]/(2[CL^{2-}]+[DPPC]) = 0.1$ (10 mol%) were prepared by the following procedure. First the corresponding amounts of DPPC and CL^{2-} solutions in methanol were mixed in a flask. Then the solvent was carefully evaporated under vacuum. A thin layer of lipid mixture was dispersed in a 10^{-2} M borate buffer, pH 9.2 with a Cole-Parmer 4700 ultrasonic homogenizer for 400 s at 55°C. SUV samples thus obtained were separated from titanium dust by centrifugation and used within 1 day.

SUVs with FITC-DPPE label incorporated into the bilayer were prepared by the same procedure, but adding a certain amount of FITC-DPPE to the lipid mixture solution before solvent evaporation. Labeled SUVs with the label content $\varphi = [\text{FITC-DPPE}]/([\text{FITC-DPPE}]+2[CL^{2-}]+[\text{DPPC}])$ equal to 0.004, 0.01 and 0.1 were thus prepared.

SUVs loaded with 1 M NaCl were prepared following the procedure described in [29]. DPPC-CL^{2-} lipid film was suspended and sonicated in 1 M NaCl 10^{-2} M borate buffer solution. Then the vesicle suspension was separated from the excess of external NaCl by passing through a column with Sephadex G-50, or dialysis against 10^{-3} M borate buffer. The integrity of NaCl-loaded SUVs was controlled by measuring conductivity of the liposome suspensions.

To determine the amount of PEVP bound to the vesicles, the reaction mixture was centrifuged for 50 min at 18 000 rpm using a J-21 centrifuge (Beckman, USA). Then the absorbance of the clear supernatant was measured at $\lambda = 257$ nm, $\varepsilon = 3400$ M^{-1} cm^{-1} using a 150/20 UV/VIS spectrophotometer (Hitachi, Japan). The concentration of PEVP in supernatant was determined using the calibration curve. By subtracting this value from the total PEVP concentration, the amount of PEVP captured by the vesicles was calculated.

2.2. Methods

The mean hydrodynamic diameter (D) of DPPC-CL^{2-} SUVs and their complexes with polyelectrolytes was determined by photon correlation spectroscopy at a fixed scattering angle (90°) in a thermostatic cell using an Autosizer IIc (Malvern, UK) equipped with a He-Ne laser. A Malvern K7032090 autocorrelator was used. The software provided by the manufacturer was employed to calculate D values. An average value over 10 consecutive measurements is reported.

The electrophoretic mobility (EPM) of SUVs and their complexes with polyelectrolytes was measured by laser microelectrophoresis in a thermostatic cell using a Zetasizer IIc (Malvern) equipped with a He-Ne laser. A Malvern K7032090 autocorrelator and the software provided by the manufacturer were also used.

Phase transitions in DPPC-CL^{2-} SUVs were registered with a DASM-4 differential adiabatic scan-

ning microcalorimeter (Design Bureau for Biological Instruments, Russia). The samples were prepared as follows. The solutions of SUVs and PEVP in a 10^{-2} M borate buffer, pH 9.2, were heated separately to 55°C and then mixed. The mixtures were kept at this temperature for 5 min, then cooled to room temperature and placed in the microcalorimeter cell. The calorimetric curves were recorded on heating the samples at a rate of 1°C/min within the range of 20–55°C.

The fluorescence intensity of the suspensions of FITC-labeled DPPC-CL^{2-} SUVs was measured at $\lambda_{em} = 525$ nm ($\lambda_{ex} = 495$ nm) using a F-4000 fluorescence spectrophotometer (Hitachi). Absorption spectra were recorded by a 150/20 UV/VIS spectrophotometer (Hitachi). The pH measurements were done using a PHM83 potentiometer with standard glass electrode 2040C (Radiometer, Denmark). The conductivity of NaCl-loaded DPPC-CL^{2-} SUVs was measured with a CDM83 conductometer (Radiometer) as described in [29].

All the experiments were performed in a 10^{-2} M borate buffer, pH 9.2. Double-distilled water used to prepare the solutions was additionally treated by passing through a Milli-Q system (Millipore, USA) equipped with ion exchange and adsorption columns and also a filter to remove large particles.

3. Results and discussion

3.1. Structural rearrangements and lipid interchange in free DPPC-CL^{2-} vesicles

Phase transition in DPPC-CL^{2-} SUVs was registered by the differential scanning microcalorimetry technique. As follows from Fig. 1 (curve 1), the original SUVs with $\nu = 0.1$, prepared by sonication of a water–lipid mixture, were characterized by a rather wide melting polytherm with a maximum at 39°C and a shoulder at 34.5°C, apparently reflecting non-uniform distribution of CL^{2-} molecules in the membrane. However, a narrowing of the calorimetric curve occurred on repeating heating and cooling cycles (curves 2 and 3). After the fourth cycle only one relatively narrow symmetrical peak with a maximum at 39°C was observed, indicating that a nearly homogeneous distribution of negative CL^{2-} molecules within the DPPC bilayer was achieved. Below 35°C the lipid bilayer was in a gel state (solid SUVs) with obviously restricted mobility of lipid molecules as a whole. Above 45°C the vesicular membrane transformed to a fluid state (liquid SUVs). Lipid molecules in such a membrane acquired lateral mobility and might migrate between the membrane leaflets (flip-flop).

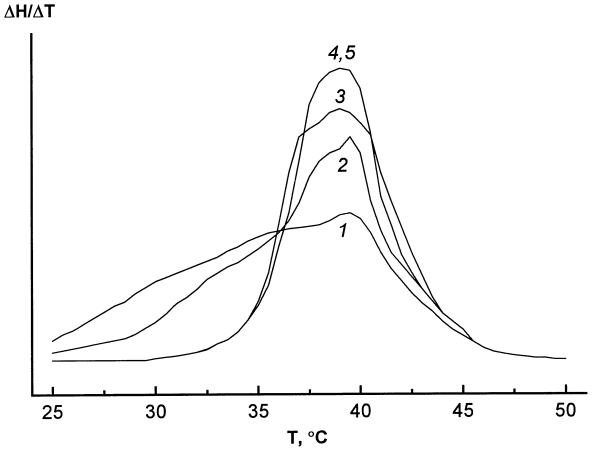


Fig. 1. Repeating records of calorimetric curves of the phase transition in DPPC-CL^{2-} SUVs (1–5). Total lipid concentration 1.0 mg/ml; 10^{-2} M borate buffer, pH 9.2.

In order to reveal the extent of lipid interchange between DPPC-CL^{2-} SUVs in gel and fluid states two samples of fluorescently labeled DPPC-CL^{2-} SUVs with FITC-DPPE incorporated into the membrane were prepared. Their φ values were equal to 0.01 and 0.1. Curves 1 and 2 in Fig. 2 represent the relative fluorescence intensities, I/I_0, of these samples at 20 and 55°C, respectively, where I_0 and I are starting and current intensity, respectively. It is seen that I/I_0 values for both SUV samples do not change for the time of observation. The lower fluorescence of the vesicles with a higher content of FITC-DPPE was apparently caused by its self-quenching within the membranes. Addition of a 9-fold excess of non-labeled DPPC-CL^{2-} SUVs to this suspension did not cause any change in fluorescence intensity at 20°C (curve 3 is consistent with curve 1 in Fig. 2). However, the fluorescence of the same mixture at 55°C increased rather rapidly approaching the level of the labeled SUVs with lower FITC-DPPE content (curve 4, Fig. 2). The only conceivable reason for such an increase was the reduction of FITC-DPPE self-quenching due to its dilution in the bilayer caused

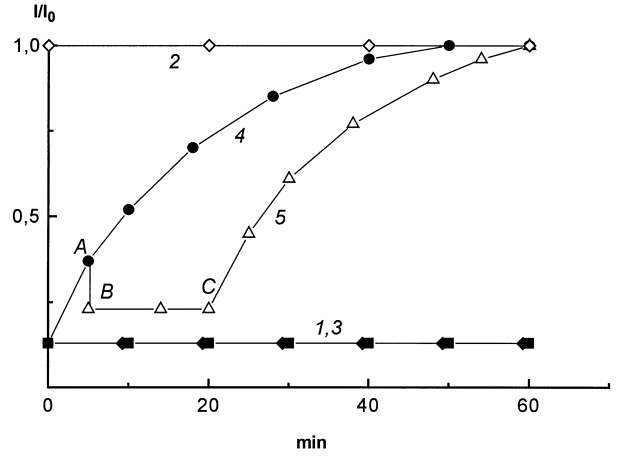


Fig. 2. Time dependence of relative fluorescence intensity of FITC-labeled DPPC-CL^{2-} SUVs ($\varphi = 0.1$) at 20°C (1), FITC-labeled DPPC-CL^{2-} SUVs ($\varphi = 0.01$) at 55°C (2), and a mixture of FITC-labeled DPPC-CL^{2-} SUVs ($\varphi = 0.1$) with DPPC-CL^{2-} SUVs (1:9 w/w) at 20 (3) and 55°C (4). Curve 5 was obtained as follows. PEVP was added to the mixture of labeled and unlabeled SUVs at point A, which resulted in a decrease in fluorescence intensity down to the level of point B. Then PAA was added to the PEVP–SUV complex at point C, [PAA]/[PEVP] = 3. In all experiments, total lipid concentration 1.0 mg/ml; [PEVP] = 1.5×10^{-4} M; 10^{-2} M borate buffer, pH 9.2.

by lipid interchange between the labeled and nonlabeled vesicles. As follows from Fig. 2, a uniform lipid distribution was established in about 1 h at 55°C. In other words, a restricted or strongly retarded lipid interchange between the solid SUVs readily occurred above their melting point. The above result correlates with earlier published data on the kinetics of lipid interchange for other SUVs depending on the phase state of their membranes [35–37].

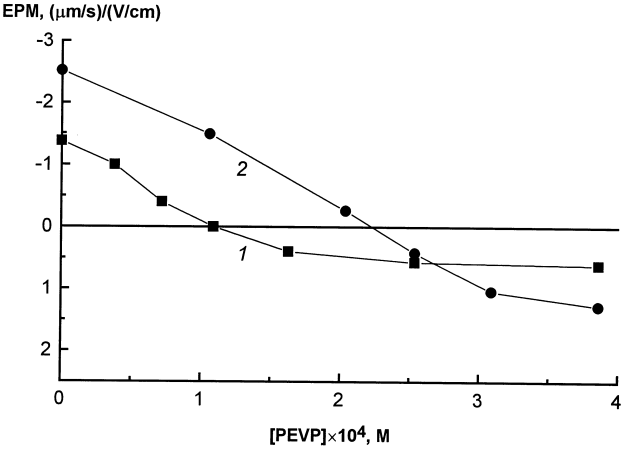


Fig. 3. Dependence of EPM of DPPC-CL^{2-} SUVs on PEVP concentration at 20 (1) and 55°C (2). Total lipid concentration 1.0 mg/ml; 10^{-2} M borate buffer, pH 9.2.

3.2. PEVP-induced structural rearrangements in the vesicular membrane

3.2.1. PEVP-induced flip-flop of lipid molecules

In earlier papers [21,28,38,39] we have already described some peculiarities of interaction of PEVP with negative mixed vesicles, in particular DPPC-CL^{2-} SUVs. The experiments were performed with solid (at 20°C) and liquid (at 55°C) SUVs. It was found that in both cases PEVP strongly bound to the vesicle surface so that about 90% of adsorbed PEVP units formed ionic pairs with CL^{2-} head groups. In both cases, adsorption of PEVP was accompanied by neutralization of the vesicle surface charge, registered by measuring the EPM of PEVP–SUV complexes, and enlargement of the light scattering particles in the system, registered by the photon correlation spectroscopy technique. The corresponding dependences are represented in Figs. 3 and 4 (curves 1 and 2). As follows from the figures, the maximum particle size was observed at EPM = 0. A further increase of the PEVP concentration resulted in overcharging the vesicle surface by the excess of

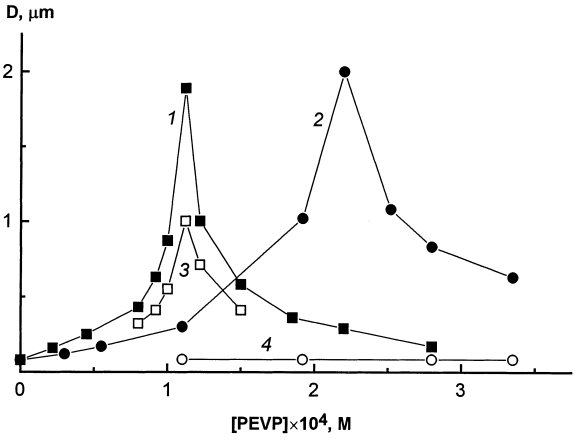


Fig. 4. Dependence of the diameter of DPPC-CL^{2-} SUVs on PEVP concentration before (1,2) and after addition of PAA (3,4) at 20 (1,3) and 55°C (2,4). Curves 3 and 4 were obtained as follows. PEVP–SUV complexes with different PEVP/SUV ratios were prepared. Then PAA was added to each complex, [PAA]/[PEVP] = 3. Total lipid concentration 1.0 mg/ml; 10^{-2} M borate buffer, pH 9.2.

adsorbed PEVP and redispersing the vesicle aggregates.

The important difference in the behavior of solid and liquid DPPC-CL^{2-} SUVs in relation to adsorbed PEVP lay in the fraction of CL^{2-} molecules forming ionic contacts with the polycation units. In the case of solid vesicles, at EPM = 0 the charge-neutralizing amount of PEVP was found to correspond to half of the total amount of CL^{2-} molecules incorporated into the membranes and homogeneously distributed between the outer and inner leaflets. In other words, only the outer negative head groups were available to form ionic contacts with PEVP. In contrast, in the case of liquid DPPC-CL^{2-} SUVs the EPM = 0 point was reached when the amount of added PEVP was twice as high as in the case of solid SUVs, i.e. equal to the total content of CL^{2-} in the membrane. Importantly, at the same time the liquid SUVs, contacting PEVP, retained their integrity as has been shown earlier [38,39]. Such behavior might be explained only by assuming an abnormally fast transfer of CL^{2-} species from the inner to outer the leaflet of the liquid vesicular membranes, induced by adsorbed PEVP (polycation-induced flip-flop). Other possible explanations such as non-complete binding of PEVP to the vesicles, or vesicular membrane disruption, or original asymmetry in charge distribution between the leaflets were rejected by the experiments and considerations described in [28]. An equal number of neutral zwitterionic DPPC species was apparently transferred to the opposite direction: from the outer to the inner membrane leaflet. Such correlated rearrangement of lipid molecules enabled the liquid DPPC-CL^{2-} membrane to retain the bilayer structure necessary to keep the vesicle integrity.

Importantly, the above difference in the behavior of solid and liquid vesicles interacting with PEVP is due to the difference in phase state and not that in temperature. In fact, it has been shown earlier that PEVP-induced flip-flop readily occurs in liquid egg lecithin-CL^{2-} SUVs at ambient temperature [28].

3.2.2. PEVP-induced lateral lipid segregation

The phase transition temperature for single component DPPC SUVs, measured by different techniques, is in the range from 41 to 41.6°C [38]. Another SUV component CL^{2-}, representing a mixture of natural lipids, is characterized by a much lower temperature (below 10°C). Therefore, the observed depression of the phase transition temperature of equilibrated mixed DPPC-CL^{2-} SUVs to 39°C (curves 4 and 5 in Fig. 1) was likely due to contamination of the DPPC bilayer with uniformly distributed CL^{2-} admixture. It also means that the microphase separation of CL^{2-} species within the mixed membrane, if it occurs, should lead to an increase of the melting point of the thus purified DPPC bilayer domains. The latter could be detected by microcalorimetric measurements.

To prepare samples for calorimetric experiments, the dispersion of DPPC-CL^{2-} SUVs and the PEVP solution were preheated over the membrane phase transition temperature and then mixed. This means that PEVP originally interacted with the liquid vesicular membranes. Then the mixture was cooled down to 20°C.

As mentioned above, the phase transition in the initial DPPC-CL^{2-} SUVs was characterized by a rather narrow peak with a maximum at 39°C (reproduced in Fig. 5, curve 1). As the concentration of PEVP added to SUV dispersion increased, the melting peak gradually shifted to higher temperatures (Fig. 5, curves 2–4) and finally superimposed on the melting peak of the single-component DPPC

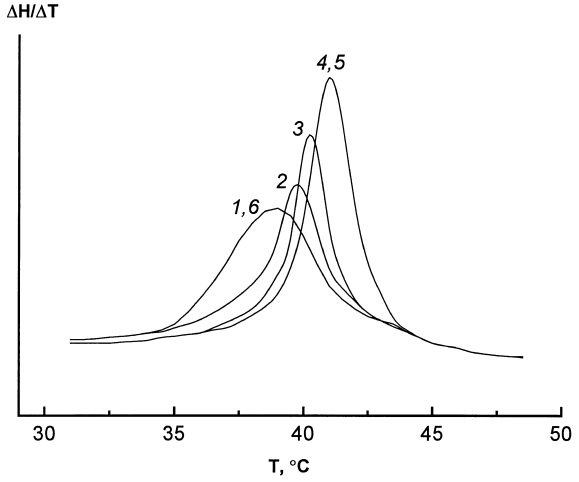


Fig. 5. Calorimetric curves of DPPC-CL^{2-} SUVs (1), their complexes with PEVP (2–4), DPPC SUVs (5), and a ternary PEVP/DPPC-CL^{2-} SUV/PAA system (6). Curve 6 was obtained as follows. A complex of PEVP with DPPC-CL^{2-} SUVs, characterized by melting curve 4, was prepared. Then PAA was added, [PAA]/[PEVP] = 3. Total lipid concentration 1.0 (1–4,6) and 0.9 mg/ml (5); [PEVP] = 5×10^{-5} (2), 1×10^{-4} (3), 2×10^{-4} M (4,6); 10^{-2} M borate buffer, pH 9.2.

vesicles (cf. curves 4 and 5 in Fig. 5). This occurred at the PEVP concentration (2×10^{-4} M) required for complete neutralization of the total amount of CL^{2-} heads incorporated into the membranes (cf. curve 2 in Fig. 3). The scope of the above data shows that PEVP-induced flip-flop in liquid DPPC-CL^{2-} SUVs is accompanied by lipid segregation, i.e. formation of CL^{2-} patches within the outer leaflet of the bilayer contacting the adsorbed polycation. Such rearrangements lead to an abnormal asymmetry in charge distribution: all negative lipid molecules concentrate on the outer membrane leaflet and form clusters, providing a maximum extent of ionic contacts with the polycations.

3.3. Removal of PEVP from the vesicular membrane

It is well known that linear polyanions form so-called interpolyelectrolyte complexes (IPECs) with linear polycations [39,40]. IPECs are stabilized by multiple ionic contacts between oppositely charged polyelectrolyte repeating units. Binding of two oppositely charged linear chains, both able to optimize their conformations within IPECs, is usually stronger than adsorption of a linear polyion on an oppositely charged rigid surface. Therefore, linear polyions (e.g. polyanions) remove electrostatically adsorbed linear polycations from surfaces, in particular from the surface of negative SUVs [19,37,41].

PEVP is a strong fluorescence quencher. Therefore, to control the extent of binding of PEVP to DPPC-CL^{2-} SUVs, we used FITC-DPPE-labeled SUVs with $\varphi = 0.004$. First, FITC-DPPE-labeled SUVs complexed with various amounts of PEVP were prepared and left to stand until the equilibrium level of fluorescence quenching was reached in these systems (approx. 1 min after mixing). PAA was then added to PEVP–SUV complex samples so that the [PAA]/[PEVP] molar ratio was equal to 3. Removal of PEVP from the vesicle surface due to recomplexation with PAA was followed by an increase in fluorescence intensity. Fig. 6 represents the equilibrium levels of fluorescence quenching caused by adsorption of PEVP on the surface of solid and liquid DPPC-CL^{2-} SUVs (curves 1 and 2), and the equilibrium levels of fluorescence recovery after addition of a 3-fold excess of PAA to the complexes of solid and liquid SUVs with PEVP (curves 3 and 4). It is seen

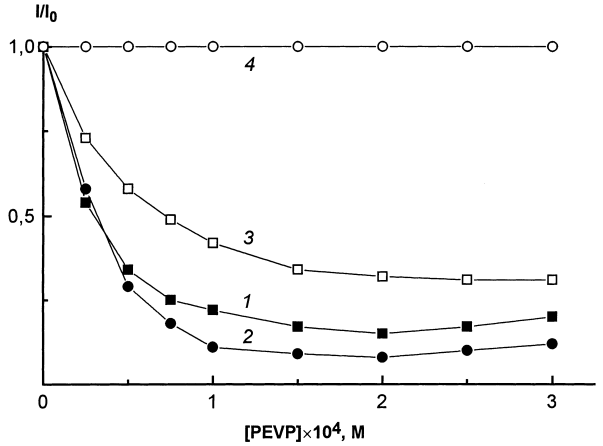


Fig. 6. Dependence of relative fluorescence intensity of FITC-labeled DPPC-CL^{2-} SUVs ($\varphi = 0.004$) on PEVP concentration before (1,2) and after addition of PAA (3,4) at 20 (1,3) and 55°C (2,4). Curves 3 and 4 were obtained as follows. PEVP–SUV complexes with different PEVP/SUV ratios were prepared. Then PAA was added to each complex, [PAA]/[PEVP] = 3. Total lipid concentration 1.0 mg/ml; 10^{-2} M borate buffer, pH 9.2.

that the addition of PAA resulted in complete recovery of the fluorescence intensity in the case of liquid SUVs. The whole process was completed within 10 s, revealing complete removal of PEVP from the surface of the liquid SUV. The removal of PEVP was accompanied by desegregation of PEVP–SUV complex particles and a decrease in their size down to that of the initial SUVs (cf. curves 2 and 4 in Fig. 4). However, PAA caused only a slight increase in fluorescence intensity when added to the complexes of PEVP with solid SUVs. This means that PAA was not able to remove PEVP from the surface of the solid SUVs. Correspondingly, in this case the particle size did not reach that characteristic of the individual SUVs (cf. curves 1 and 3 in Fig. 4).

Such a difference in the behavior of complexes of PEVP with solid and liquid SUVs in the presence of PAA looks rather astonishing. Indeed, as shown above, binding of PEVP to liquid DPPC-CL^{2-} SUVs has been accompanied by clustering of all CL^{2-} species within the outer membrane leaflet, i.e. resulted in the formation of a maximum number of ion pairs responsible for PEVP-CL^{2-} complexation at the SUV surfaces. One may expect that such a complex should be stronger than that of PEVP with solid SUVs where ion pair adjustment is hin-

dered by a lack of lipid mobility. However, the reverse is true in practice.

The reason for this discrepancy might be as follows. It is known that below the phase transition temperature the probability of an accumulation of defects in the vesicular membrane increases because of the decrease in their curing rate. Such an accumulation can be accompanied even by a subsequent fusion of vesicles [42–44]. We have no direct evidence that individual solid DPPC-CL^{2-} SUVs have been actually fused under our experimental conditions. However, adsorption of PEVP, followed by SUV aggregation, could also produce some perturbations in the solid membranes non-cured below the phase transition temperature. As a result, PEVP chains could partially incorporate into the newly formed bilayers and become not only electrostatically adsorbed but also sterically trapped. Then they could not be removed from the complexes by recomplexation with added PAA.

The formation of defects in the membrane of solid DPPC-CL^{2-} SUVs upon interaction with PEVP was experimentally confirmed by an increase in ionic permeability of the membrane. A suspension of solid SUVs filled with 1 M NaCl solution was prepared and mixed with a PEVP solution. Then the conductivity of the suspension was measured. The obtained value was compared with the ultimate conductivity measured after irreversible disruption of SUVs on their treatment with a 10-fold excess of a Triton X-100 solution resulted in the complete release of NaCl originally captured inside the SUVs. It was found that PEVP binding to NaCl-filled solid SUVs was followed by a 30–35% increase in the suspension conductivity with respect to its ultimate level. (PEVP solution was also added to the suspension of NaCl-filled liquid DPPC-CL^{2-} SUVs. No increase in the suspension conductivity was observed in this case, indicating that the liquid vesicular membrane retained its low permeability for the simple ions.) This means that adsorbed PEVP has actually produced some defects in the solid vesicular membrane responsible for a partial release of the salt. Importantly, this salt efflux was not accompanied by irreversible disruption of the membranes. It has been shown earlier [45–47] that polycation-induced fusion of vesicles is often accompanied by partial leakage of the water-soluble contents from the vesicle interior to the outer solution. Therefore, the hypothetical possibility of PEVP-induced fusion of the solid DPPC-CL^{2-} SUVs cannot be excluded either.

3.4. Structural recovery of the liquid vesicular membrane after PEVP removal

As described above, complexation of PEVP with liquid DPPC-CL^{2-} SUVs produced enormous asymmetry in the charge distribution within the membrane. A natural question arises: whether the original homogeneous distribution of the lipids in the membrane is restored after removal of the polycation.

The results in Fig. 7A show a change of the EPM of DPPC-CL^{2-} SUVs with time at 55°C after removal of PEVP from the membrane by the addition of PAA. Point A in the figure corresponds to the EPM of the original liquid DPPC-CL^{2-} SUVs with nearly homogeneous distribution of CL^{2-} between both leaflets of the membrane. It should be emphasized that only the outer CL^{2-} (i.e. half of the total CL^{2-} molecules) contribute to the surface vesicle charge, determining the EPM value of the original SUVs. Point B corresponds to the EPM of SUVs whose surface charge has been neutralized by adsorbed PEVP. One minute after the addition of PAA to the PEVP–liquid SUV complex, negative particles were registered in the system characterized by an EPM value approximately twice as high as the EPM of the original SUVs (point C). The size of these negative particles, measured by photon correlation spectroscopy, was equal to that of the original SUVs. The EPM of the particles gradually decreased and in approx. 30 min reached the EPM value of the original SUVs (curve 1).

As shown above, PAA completely removed PEVP from the membrane of liquid DPPC-CL^{2-} SUVs within approx. 10 s. This means that the EPM value, registered 1 min after PAA addition to the PEVP–SUV complex, actually corresponded to the EPM of the liquid DPPC-CL^{2-} SUVs already free of adsorbed PEVP. At this moment, the membrane was asymmetric to the utmost: nearly all negative CL^{2-} molecules were still located on the outer leaflet of the membrane. Correspondingly, the surface charge and, therefore, the EPM of such SUVs were twice as high as those of the original SUVs. In the course of time,

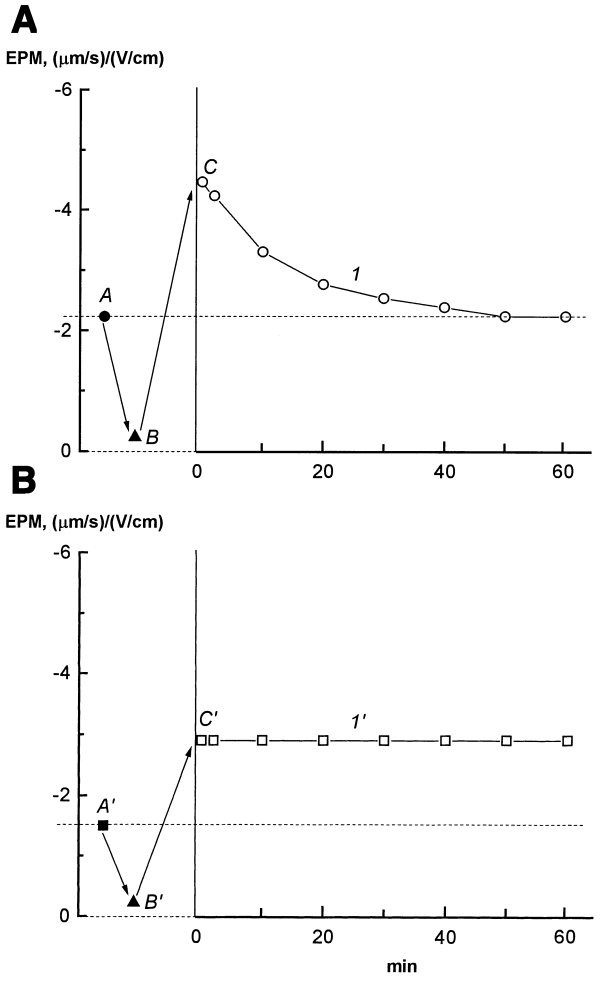


Fig. 7. (A) EPM values of free liquid DPPC-CL^{2-} SUVs (point A), liquid SUVs complexed with PEVP (point B), and PEVP–liquid SUV mixture after addition of PAA (point C), [PAA]/[PEVP] = 3. Curve 1 describes the EPM decrease with time after removal of PEVP. Total lipid concentration 1.0 mg/ml; 10^{-2} M borate buffer, pH 9.2; 55°C. (B) EPM values of free solid DPPC-CL^{2-} SUVs (point A′), solid SUVs complexed with PEVP (point B′), and PEVP–solid SUV mixture after addition of PAA (point C′), [PAA]/[PEVP] = 3. The EPM of the PEVP–solid SUV–PAA mixture vs. time is given by curve 1′. Total lipid concentration 1.0 mg/ml; 10^{-2} M borate buffer, pH 9.2; 20°C.

half of the CL^{2-} molecules were transferred from the outer to the inner membrane leaflet, decreasing the EPM to that of the original SUVs.

The reversibility of the structural rearrangements in the liquid vesicular membrane, induced by adsorption/desorption of PEVP, was also demonstrated by microcalorimetric measurements. The experiments were done as follows. The 1 mg/ml suspension of DPPC-CL^{2-} SUVs, preheated to 55°C, was treated with a 2×10^{-4} M PEVP solution so that the PEVP amount was sufficient to neutralize all CL^{2-} heads incorporated into the membrane, indicating the complete lipid segregation described above. The latter follows from coalescence of the melting peaks at the calorimetric curves in Fig. 5: one corresponding to the PEVP–DPPC-CL^{2-} SUV complex (curve 4), and another to SUVs consisting of pure DPPC (curve 5). Then 3-fold excess of PAA with respect to PEVP was added to the PEVP–SUV complex to remove the polycation from the membrane. The obtained mixture was kept at 55°C for 30 min, then cooled to room temperature, and the calorimetric curve was recorded (Fig. 5, curve 6). It is seen that this curve coincides completely with curve 1 obtained for the original DPPC-CL^{2-} SUVs. This result, together with the above EPM change for liquid SUVs, shows that removal of PEVP from the liquid membrane is accompanied by complete recovery of the original lateral and transmembrane distribution of the lipids.

As to solid DPPC-CL^{2-} SUVs, the changes in their EPM after complexation with PEVP and further addition of PAA are represented in Fig. 7B. Point A′ corresponds to the EPM of the original solid SUVs, and point B′ to the EPM of solid SUVs complexed with PEVP. Addition of PAA to the PEVP–SUV complex resulted in the formation of negative particles whose EPM was approx. 1.5 times higher than the EPM of the original solid SUVs (point C′). Importantly, the established EPM value did not change with time (curve 1′). As shown above, PAA was not able to extract PEVP from its complex with solid SUVs. Therefore, the pattern in Fig. 7B most likely reflects adsorption of PAA on PEVP–SUV complex particles, i.e. formation of a ternary PEVP–SUV–PAA complex whose negative surface charge has been brought about by the excess of the polyanion units exposed to the aqueous phase.

3.5. *Effect of PEVP adsorption on the intervesicular lipid exchange*

In order to explore whether adsorption/desorption of PEVP influences the exchange of lipid molecules between liquid SUVs, the fluorescence method has been applied. First, FITC-labeled DPPC-CL^{2-}

SUVs with a self-quenching content of FITC label ($\varphi = 0.1$) were mixed with unlabeled DPPC-CL^{2-} SUVs. As described in Section 1, an increase in FITC fluorescence intensity was observed just after mixing, caused by the redistribution of FITC-DPPE molecules between all SUVs in the system and the dilution of FITC in the vesicular membranes (see Fig. 2, curve 4).

Fig. 2 also shows that addition of PEVP to the mixture of labeled and unlabeled DPPC-CL^{2-} SUVs at point A resulted in a decrease in FITC fluorescence intensity down to the level marked by point B in curve 5. The established fluorescence level remained unchanged while PEVP kept contacting the SUV surface (region between points B and C in curve 5). Addition of PAA (at point C), causing the removal of PEVP from its complex with SUVs, was followed by a gradual increase in fluorescence intensity. The ultimate value was reached in about 40 min.

Two important conclusions follow from these results. First, PEVP adsorption strongly terminates the interchange of negative FITC-DPPE and probably CL^{2-} species between the liquid SUVs, most likely because of their involvement in the electrostatic complex with PEVP units. Second, removal of PEVP from the membrane is accompanied by complete recovery of the lipid interchange between vesicles.

4. Conclusions

The data obtained allow to suggest the following mechanism of interaction of the synthetic polycation with the negative vesicles.

Complexation of PEVP with solid DPPC-CL^{2-} SUVs is accompanied by the formation of defects in the membrane and most likely SUV fusion. Such complexation in addition to electrostatic interaction is probably contributed by hydrophobic interaction. Therefore, adsorbed PEVP polycations keep contacting solid SUVs even in the presence of an abundant amount of PAA polyanions.

It was shown earlier that the interaction of PEVP with liquid DPPC-CL^{2-} SUVs led to drastic structural rearrangements in the vesicular membrane. Negative CL^{2-} species migrated from the inner to the outer membrane leaflet and apparently concentrated in the vicinity of adsorbed PEVP chains. Both processes resulted in an abnormal asymmetry in charge distribution within the liquid vesicular membrane. At the same time, the integrity of liquid SUVs contacting PEVP was completely retained. Since the interaction of PEVP with liquid SUVs was electrostatic in nature, the polycation could be completely removed from the vesicular membrane by recomplexation with an excess of PAA. In this work we have demonstrated that PEVP adsorption also terminates the exchange of lipid molecules between SUVs. Removal of PEVP by adding PAA results in the complete resumption of the original distribution of lipids in lateral and transmembrane directions. The lipid exchange between SUVs also recovers. However, the recovery proceeds much slower than the removal of PEVP. It may offer the possibility to fix the above asymmetry in charge distribution in free SUVs by quick tempering below the phase transition temperature.

It is known that membranes of living cells in a biological environment are usually in the liquid (fluid) state and negatively charged. One can assume that polycations interacting with living cells may also produce a significant charge asymmetry in a lipid bilayer and also prevent lipid molecules from intercell migration. At the same time, cell membrane integrity may be retained, and adsorbed polycations removed from the membrane by recomplexation with competing polyanion species, resuming the initial cell membrane structure and intercell mobility of lipids. The established phenomena may contribute to understanding the biological effects of polycations.

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